

"Express Mail" mailing label number: EV003894909US

Date of Deposit: December 14, 2001

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. §1.10 on the date indicated above and is addressed to BOX PATENT APPLICATION, Commissioner for Patents, Washington D.C. 20231.

Typed or Printed Name of Person Mailing Paper or Fee: Patricia Aguillon

Signature: Patricia Aguillon

PATENT
Docket No. P1384

PATENT APPLICATION

INCUBATION METHOD FOR OBTAINING SOLID CULTURE OF ZANG ZHI, SOLID CULTURE OBTAINED THEREFROM, PROCESSED PRODUCTS AND USE THEREOF

INVENTORS: MING-HUANG LAN
 LI-YU WU

BACKGROUND OF THE INVENTION

[0001] Zang Zhi is a kind of fungus. Its academic name is *Antrodia camphorata*, also known as "Zang ku", "red Zang Zhi", "niou Zang Zhi" or "niou Zang ku." In Taiwan, Zang Zhi only grows on the inner wall of the empty rotting trunk of *Cinnamomum kanehirai* in the shape of a platy or bell and has a strong cinnamon flavor. People say that Zang Zhi can tranquilize, prevent or treat a cold, promote vital energy circulation, remove blood stasis, promote blood circulation, relieve dyspepsia, detoxify and promote the subsidence of swelling, calm the nerves to reduce stress, alleviate pain, and is the best anticorrosive and detoxicant. It is considered the most expensive wild fungus in the market on Taiwan. The mechanism of anti-tumor cells of Zang Zhi is different from that of *Ganoderma* sp., which indirectly promotes immunity against tumors. Zang Zhi can directly inhibit or kill cancer cells, and has the capacity to strengthen the heart, adjust immunity, and antagonize the parasympathetic nervous system, and has serous activity. In particular, Zang Zhi can cure a stomachache, bowelache, nausea, diabetes, gout, arthritis, fever, allergy, exorbitant urine proteins, uremia, cirrhosis, hepatoma, flu, etc. Many research institutions have invested human resources and materials in the study of the active constituents of Zang Zhi. Among them, the National Science Council of the Executive Yuan has a special research project to make use of chemical methods, NMR spectrum analysis, and comparative spectrum with

known materials to establish the constitutive characteristics of active constituents. Research results of the active constituents show that the water and methanol extracts of Zang Zhi are effective on the inhibition of the growth of *Streptococcus aureus* and *Trichophyton mentagrophytes*. Zhankuic acid A, a methanol extract of Zang Zhi has conspicuous inhibition to the lymphoma cells of a p-388 mouse and agglutination of blood platelets. Zhankuic acid B, however, shows little anticholinergic and anti-serotonin effect.

[0002] It is indicated in Gau Sheau Jy's masters thesis on the triterpenoid of Zang Zhi that Zang Zhi is a new species of *Ganodenna* discovered in 1990. The extract of the solid fruiting body is obtained by use of acetone, and then is separated and re-crystallized with chromatography LC and PLC. The purity of the extract is identified by TLC scanning and HPLC. The configuration test is done with mass spectrum, infrared spectrum, ultraviolet spectrum, H-NMR, and C-NMR. 3,11- dioxo-8,23-dien-26-oic acid can reduce GPT in the blood of a mouse with the acute hepatitis induced by CCl₄.

[0003] Most of Zang Zhi grows in the broad-leaf forest areas in Taiwan's East Coast Mountain Range. However, according to the bulletin of the Niou Zang Conservation Notice issued by the Taiwan Forestry Bureau, this mountain range has been listed as a natural resources conservation zone. Since Zang Zhi is a very popular product which most people are willing to purchase in Asia, Zang Zhi growing outside the said zone have been almost completely harvested. In fact, wild Zang Zhi are not available in the local markets.

[0004] With an estimated sale of 500 kg Zang Zhi per month in Taiwan, the requirement to provide an incubation method for solid culture of Zang Zhi has become more important in recent years.

SUMMARY OF THE INVENTION

[0005] The main objective of the subject invention is to provide an incubation method for solid culture of Zang Zhi. The cultured Zang Zhi will have the same pharmaceutical efficacy as the wild one does.

[0006] The other objective of this invention is to provide a solid culture of Zang Zhi by use of the inoculums of Zang Zhi spawn preserved in the Food Industry Research and Development Institute (Taiwan), coded CCRC 35398, to incubate the solid culture in accordance with the incubation method.

[0007] Another objective of the invention is to provide the processed products of the solid cultured Zang Zhi.

[0008] Another objective of the invention is to provide a solid cultured Zang Zhi useful as active constituents.

BRIEF DESCRIPTIONS OF THE DRAWINGS

[0009] Fig. 1 illustrates comparative analysis of the HPLC of constituents of a Zang Zhi, including (1) the fruiting body of cultured Zang Zhi according to the invention, (2) the liquid culture of Zang Zhi and (3) the fruiting body of wild Zang Zhi.

[0010] Fig. 2 illustrates the use of ferrous ions to stimulate the homogenization of mouse brain to cause the free radical peroxidative reaction of lipid, which will result in increasing of the TBARS (peroxidative constituents of lipid). Comparing the triple fold with the eight fold of concentrated extract of Zang Zhi fruiting body cultured according to the invention, we note that the increase inhibition of the peroxidative reaction will vary with the increased of the concentration, that is shown by the percentage of inhibition of per oxidative reaction (n=3).

[0011] Fig. 3 shows the effect of different concentrations of extract of Zang Zhi on the active change of GTP for measurement of liver function. # represents the statistical difference between the normal group and the injury group ($P < 0.01$); *represents the statistical difference between the feed group and the injury group ($P < 0.01$). Value is represented by average \pm standard error.

[0012] Fig. 4 shows the effect of different concentrations of Zang Zhi extract on the active change of GOT for the biochemical measurement of liver function. # represents the statistical difference between a normal group and an injury group ($P < 0.01$); *represents the statistical difference between the feed group and the injury group ($P < 0.01$). Value is represented by average \pm standard error.

[0013] Fig. 5 shows the effect of an extract of Zang Zhi powder on the growth of bowel cancer cell (COLO 320 HSR).

DETAILED DESCRIPTION OF THE INVENTION

[0014] This invention provides a method for the solid culture of Zang Zhi, in order to produce the Zang Zhi fruiting body with the constituents and vitality of wild Zang Zhis. The invention's method for the solid culture of Zang Zhi is to culture the spawn of Zang Zhi through "Bag Log" cultivation, and then to produce the fruiting body of Zang Zhi in the air.

[0015] Before the phase of mycelium culture, it is usual to greatly multiply the spawn for "Bag Log" cultivation, including the following three steps: (1) taking a piece of medium agar containing hyphae preserved in liquid nitrogen, and transfer it into a fresh medium to culture under constant temperature until the exuberant growth of mycelium appears; (2) inoculating the spawn into the "Bag Log" containing cellulolytic substance (for example kernel or spelk); (3) removing the supra old hyphae until the hyphae overgrows in the "Bag Log"; and (4) then inoculating the multiplied spawn into "Bag Log".

[0016] Before the phase of mycelium culture, one also can greatly multiply spawn with the liquid culture fermentation, and then inoculate them into "Bag Log". The formulation of liquid culture fermentation is 1-3% of fructose, 0.01-0.1% of magnesium sulfate, 0.1-1% of yeast extract, and 0.05-0.5% of potassium phosphate. The best formulation of liquid culture fermentation followed was 2% of fructose, 0.05% of magnesium sulfate, 0.5% of yeast extract, and 0.1% of potassium phosphate.

[0017] After culturing the slide tube, one may inoculate the multiplied spawn into a 5 liter liquid culture for fermentation under 24-26°C, 240 rpm oscillation for 14 days, and then 90 rpm oscillation for 14 days, and then inoculate them into a 20 liter liquid fermentation, stirring culture for 14 days.

[0018] Here, the so-called "Bag Log" is the plastic bag made from 10-70% of cellulolytic material of stem, stalk, fruit, or spelk of any mushroom or plant (especially, the stems, stalks, and fruits of grass plants or even cellulolytic spelk are better), 10-30% of starch resource (especially, potato is better), and 5-15% of millet (rice bran is better), 1-10% of saccharides (glucose is better), 0.5-2% of phosphate (potassium phosphate is better), and 0.1-1% of sulfate salt (magnesium sulfate is better). Relative humidity is maintained at 60-90%, better at 80%. The pH value of the culture medium is adjusted to be neutral.

[0019] The phase of mycelium culture indicated in this text means a period of sixty days after the spawn is inoculated into "Bag Log". Mycelium will grow at a temperature from 5° to 32°C; in particular, 28°C is the best temperature for the growth of mycelium. "Bag Log" cultivation is under way at a relative humidity of about 60-80%, and better at about 80%. As to the condition of air, 0.1-1% of carbon dioxide is better for the culture.

[0020] After the phase of mycelium culture, the phase of the culture of the fruiting body is next. That is to say 61-90 days after the date of the inoculation of the spawn, the plastic bag of "Bag Log" is to be removed, to let the sawdust medium be completely exposed to the air. During the phase of the culture of the fruiting body, the difference between day and night temperature has to be under strict control. Day temperature is generally maintained between 20° and 30°C, especially, better at 25±1°C; night temperature is better maintained at 11±1°C; the difference between day and night temperature is better maintained at 15°C. The relative humidity at the phase of fruiting body culture is controlled between 90 and 95%. In addition, the fruiting body has to be cultured in the moving air, and the carbon dioxide is less than 1%. Generally, solid culture material will be collected after 90-120 days.

[0021] The strain CCRC35398 spawn used in this invention can be obtained from the Food Industry Research and Development Institute, Taiwan. According to the solid culture method of this invention, a solid culture substance was obtained. The solid culture substance is conoid, about 10-30 cm in diameter, 15-30 cm in height, and 0.2-0.6 kg in weight.

[0022] To use the solid culture method described above, one can get the solid Zang Zhi. The solid Zang Zhi can be produced by any of the processing methods known to those skilled in the art, for example, water or alcohol can be used to extract the active constituents, and then known concentration methods such as vacuum concentration or freeze concentration can be used to produce the concentrated product.

[0023] One can use the dehydrated method to do the dehydration, or by a spray-dry or lyophilization method. However, the lyophilization method is the best one to dehydrate the solid substance, and during dehydration the solid substance will not change its characteristic by the effect of oxygen.

[0024] The microscopic particles produced by the spray-dry or other dehydration method can be agglomerated to increase their diameters. These products are poremeric particles with good wettability.

[0025] The comparative HPLC spectrum of the invention's solid culture Zang Zhi, known liquid cultured Zang Zhi, and wild Zang Zhi is shown in Figure 1. By analysis of HPLC, the constituents of the fruiting body obtained through the invention's culture method are similar to those of the wild Zang Zhi and the invention's Zang Zhi has the activities and functions similar to the wild Zang Zhi's. These activities and functions include tranquilization, prevention from or treatment of a cold, promoting vital energy circulation, removing blood stasis, promoting blood circulation, relieving dyspepsia, detoxifying and promoting the subsidence of swelling, calming the nerves to reduce stress, alleviating pain, inhibiting bacteria and virus and cancer cells, strengthening the heart, adjusting immunity, and antagonizing the parasympathetic nervous system and serous activity. Zang Zhi can cure a

stomachache and bowel ache, nausea, diabetes, gout, arthritis, infection, allergy, exorbitant urine proteins, uremia, cirrhosis, hepatoma, flu and so on. Zang Zhi can regenerate skin and act as human skin or callus material, or as adhesive for bedsore or traumatic skin.

[0026] Furthermore, the invention's solid cultured substance possesses the abilities to antagonize the free radicals and peroxidation of lipids, and functions to protect the liver and inhibit cancer cells. The invention's solid cultured substance can be taken as health food to prolong life, and be further developed into new medicines.

[0027] The following working examples will further explain this invention, but not limit this invention. Any application or modification of this invention made by persons skilled in these techniques will fall in the scope of this invention.

Working Examples

[0028] The strain CCRC35398 spawn obtained from the Food Industry Research and Development Institute were grown by ferment liquid culture, and then inoculated into the "Bag Log". The fermented liquid culture contains 2% of fructose, 0.05% of magnesium sulfate, 0.5% of yeast extract, and 0.1% of potassium phosphate. "Bag Log" cultivation is to culture mycelium. "Bag Log" is composed of 65% of the stems, stalks, fruits of grass plants or cellulolytic spelk, 20% of potato, 10% of rice bran, 3.5% of glucose, 1% of potassium phosphate, and 0.5% of magnesium sulfate. Adjust the relative humidity to 80% and pH to 7. Relative humidity was maintained at 60-90%, better at 80%, and the pH of the culture medium was adjusted to being neutral.

[0029] The period of mycelium culture is 60 days after the spawn was inoculated into "Bag Log". Mycelium will grow at a temperature between 5° and 32°C; 28°C is the optimal temperature for the growth of mycelium. "Bag Log" humidity is at 80%, and air condition is 0.2-1% of carbon dioxide.

[0030] After the phase of the mycelium culture, the phase of the culture of the fruiting body is on, that is to say 61-90 days after the date of inoculation of the spawn, the plastic bag of "Bag Log" is to be removed, to let the sawdust medium be completely exposed in the air. During the phase of the culture of the fruiting body, the difference between day and night temperature has to be under strict control. Day temperature is generally maintained between 20° and 30°C; night temperature is better maintained at 11±1°C; the difference between day and night temperature is better maintained at 15°EC. The relative humidity of the phase of the fruiting body culture is controlled between 90 and 95%. In addition, the fruiting body has to be cultured in the moving air, and the carbon dioxide is less than 1%.

[0031] In 90-120 days, when the conoid fruiting body grows up to 15 cm in diameter and 25 cm in height, the solid-cultured substance will be collected, and then the substance is about 0.4 kg in weight. The HPLC spectrum of major active elements - triterpenoid - is shown in Figure 1(1), and the measurements are shown in the following Table 1.

Table 1, Data of HPLC analysis of the invention's Zang Zhi fruiting body

Table 1

No.	retention time	area (uv*sec)	area%	relative height	height%	conc	conc unit	baseline	ingredient name
1	2.060	27958870	9.3018	1000.279	6.5362	0.0000		vv	
2	2.547	6320387	2.1028	999.303	6.5299	0.0000		vv	
3	2.663	6333234	2.1070	999.158	6.5289	0.0000		vv	
4	2.823	13521140	4.4984	998.886	6.5271	0.0000		vv	
5	3.117	3933511	1.3087	328.263	2.1450	0.0000		vv	
6	3.413	4614312	1.5352	303.141	1.9808	0.0000		vv	
7	3.673	2519874	0.8384	182.128	1.1901	0.0000		vv	
8	3.973	2084553	0.6935	144.477	0.9441	0.0000		vv	

9	4.367	4265658	1.4192	149.458	0.9766	0.0000	VV
10	5.150	7601131	2.5289	460.320	3.0079	0.0000	VV
11	5.773	1671478	0.5561	71.272	0.4657	0.0000	VB
12	6.043	441485	0.1469	54.184	0.3541	0.0000	BV
13	6.360	1264441	0.4207	62.749	0.4100	0.0000	VV
14	6.597	1359149	0.4522	58.680	0.3834	0.0000	VV
15	7.263	1946283	0.6475	56.407	0.3686	0.0000	VV
16	7.660	517391	0.1721	43.081	0.2815	0.0000	VV
17	8.167	5862269	1.9504	317.837	2.0769	0.0000	VV
18	8.983	932610	0.3103	29.915	0.1955	0.0000	VV
19	9.990	1253931	0.4172	34.691	0.2267	0.0000	VV
20	10.343	538013	0.1790	22.899	0.1496	0.0000	VV
21	10.823	164318	0.0547	12.337	0.0806	0.0000	VV
22	11.327	1233415	0.4104	29.716	0.1942	0.0000	VV
23	12.193	628302	0.2090	19.258	0.1258	0.0000	VV
24	12.990	1259055	0.4189	37.378	0.2442	0.0000	VV
25	14.810	2982581	0.9923	80.403	0.5254	0.0000	FP
26	17.313	2521967	0.8390	68.342	0.4466	0.0000	ZV
27	18.113	627346	0.2087	19.934	0.1303	0.0000	VV
28	18.990	117339	0.0390	4.690	0.0306	0.0000	VV
29	19.867	663514	0.2207	20.198	0.1320	0.0000	VV
30	20.867	2932057	0.9755	52.760	0.3448	0.0000	VV
31	22.323	252149	0.0839	9.636	0.0630	0.0000	VV
32	23.080	3131662	1.0419	79.476	0.5193	0.0000	VV
33	23.960	1130832	0.3762	31.023	0.2027	0.0000	VV
34	25.857	4135840	1.3760	78.025	0.5098	0.0000	VV
35	26.910	1070268	0.3561	22.420	0.1465	0.0000	FP
36	28.940	2127664	0.7079	44.217	0.2889	0.0000	VV
37	30.503	15997700	5.3224	304.306	1.9885	0.0000	VZ
38	33.793	5256445	1.7488	92.365	0.6036	0.0000	FP
39	35.373	462920	0.1540	8.405	0.0549	0.0000	VV
40	37.360	4416830	1.4695	79.089	0.5168	0.0000	VZ
41	39.193	261669	0.0871	5.522	0.0361	0.0000	FP
42	41.267	4411946	1.4678	73.326	0.4791	0.0000	VV
43	42.667	2821209	0.9386	50.936	0.3328	0.0000	VV
44	44.323	36608	0.0122	1.071	0.0070	0.0000	FP
45	46.630	10087950	3.3562	134.950	0.8818	0.0000	FP
46	50.090	333401	0.1109	4.502	0.0294	0.0000	FP
47	52.383	4896649	1.6291	60.376	0.3945	0.0000	VV
48	55.280	124357	0.0414	2.047	0.0134	0.0000	FP
49	56.593	4986938	1.6591	340.488	2.2249	0.0000	FP
50	56.953	7314754	2.4336	625.513	4.0874	0.0000	VV
51	57.290	4586732	1.5260	463.198	3.0267	0.0000	VV
52	57.800	3699586	1.2308	189.756	1.2399	0.0000	VV
53	58.087	1056724	0.3516	118.120	0.7718	0.0000	VV
54	58.477	2468879	0.8214	134.218	0.8770	0.0000	VV
55	58.690	1982234	0.6395	157.326	1.0293	0.0000	VV
56	58.927	1445816	0.4810	124.837	0.8157	0.0000	VV
57	59.210	3138696	1.0442	171.327	1.1195	0.0000	VV

85	73.070	513554	0.1709	13.848	0.0905	0.0000	VV
86	73.730	419796	0.1397	9.953	0.0650	0.0000	VV
87	74.950	357733	0.1190	12.698	0.0830	0.0000	VV
88	75.373	465804	0.1550	15.474	0.1011	0.0000	VV
89	76.253	356155	0.1185	10.162	0.0664	0.0000	VV
90	77.137	133340	0.0444	4.645	0.0304	0.0000	VV
91	77.850	272953	0.0908	11.805	0.0771	0.0000	VV
92	78.420	752946	0.2505	30.824	0.2014	0.0000	VV
93	78.690	869866	0.2894	31.809	0.2079	0.0000	VV
94	79.653	66206	0.0220	2.235	0.0146	0.0000	VP
95	80.730	79648	0.0265	2.862	0.0187	0.0000	PP
96	81.753	27502	0.0091	1.070	0.0070	0.0000	PP
97	83.147	186611	0.0621	4.143	0.0271	0.0000	PP
98	83.923	293714	0.0977	9.081	0.0593	0.0000	VV
99	84.520	361025	0.1201	9.061	0.0592	0.0000	VV
100	85.820	820423	0.2730	29.144	0.1904	0.0000	VV
101	86.260	1531752	0.5096	35.708	0.2333	0.0000	VV
102	87.803	1945022	0.6471	51.978	0.3396	0.0000	VV
103	89.500	271109	0.0902	7.353	0.0480	0.0000	VP
104	90.883	26832	0.0089	0.533	0.0035	0.0000	PP
105	92.080	55385	0.0184	1.398	0.0091	0.0000	PP
106	96.443	3758662	1.2505	52.867	0.3455	0.0000	PP
107	100.237	94086	0.0313	1.137	0.0074	0.0000	PP
108	106.650	1435702	0.4777	41.107	0.2686	0.0000	PP
109	107.300	1158655	0.3855	55.895	0.3652	0.0000	VV
110	107.720	1487062	0.4947	71.435	0.4668	0.0000	VV
111	108.190	709049	0.2359	37.254	0.2434	0.0000	VV
112	108.493	818254	0.2722	38.627	0.2524	0.0000	VV
113	109.013	631416	0.2101	35.016	0.2288	0.0000	VV
114	109.507	1010362	0.3361	33.653	0.2199	0.0000	VV
115	109.900	446664	0.1486	27.863	0.1821	0.0000	VV
116	110.160	598899	0.1993	33.360	0.2180	0.0000	VV
117	110.493	840683	0.2797	57.202	0.3738	0.0000	VV
118	111.023	2135668	0.7105	103.717	0.6777	0.0000	VV
119	111.450	547756	0.1822	42.198	0.2757	0.0000	VV
120	111.833	1597280	0.5314	56.890	0.3717	0.0000	VV
121	112.473	967994	0.3220	55.981	0.3658	0.0000	VV
122	112.607	679050	0.2259	59.614	0.3895	0.0000	VV
123	112.820	536941	0.1786	49.913	0.3261	0.0000	VV
124	113.067	987108	0.3284	35.926	0.2348	0.0000	VV
125	113.807	1383823	0.4604	34.665	0.2265	0.0000	VV
126	114.563	1002529	0.3335	36.357	0.2376	0.0000	VV
127	114.970	520199	0.1731	24.136	0.1577	0.0000	VV
128	115.540	1143580	0.3805	28.119	0.1837	0.0000	VV
129	116.577	338258	0.1125	11.209	0.0732	0.0000	VV
130	117.017	176187	0.0586	8.880	0.0580	0.0000	VV
131	117.400	169393	0.0564	8.586	0.0561	0.0000	VV
132	118.133	518740	0.1726	11.014	0.0720	0.0000	VV
133	118.763	134439	0.0447	7.091	0.0463	0.0000	VV
134	119.237	368278	0.1225	10.127	0.0662	0.0000	VP
135	121.140	64584	0.0215	2.560	0.0167	0.0000	PP
136	121.620	185059	0.0616	6.282	0.0410	0.0000	VV
137	122.620	30314	0.0101	1.935	0.0126	0.0000	VV
138	123.090	254238	0.0846	8.468	0.0553	0.0000	VV
139	124.110	141072	0.0469	6.206	0.0406	0.0000	VP
140	125.877	378418	0.1259	7.976	0.0521	0.0000	PP
141	127.977	27554	0.0092	1.016	0.0066	0.0000	PP
142	129.813	187815	0.0625	9.937	0.0649	0.0000	PP
total		300574826	15303.60	0.0000			

[0032] The differences between the invention's Zang Zhi fruiting body, other cultural methods and liquid cultural method were compared by the analysis of HPLC. The HPLC spectra-are shown in Figure 1(2) -(3); and the measurements are shown in Table 2 and Table 3. These spectra show that the invention's Zang Zhi fruiting body and wild Zang Zhi have the same shape of peak, and also show that the invention's cultured Zang Zhi fruiting body and that of wild Zang Zhi have the same activities and functions.

Table 2. HPLC spectrum figure of liquid cultured Zang Zhi

Table 2

No.	retention time	area (uv*sec)	area%	relative height	height%	conc	conc unit	baseline	ingredient name
1	1.620	262998	1.2184	38.745	2.2045	0.0000		PV	
2	1.857	8294342	38.4242	660.685	37.5912	0.0000		VV	
3	2.520	5417476	25.0969	726.398	41.3301	0.0000		VV	
4	2.820	1592534	7.8413	99.406	5.6559	0.0000		VV	
5	3.790	731157	3.3871	23.209	1.3205	0.0000		VB	
6	4.123	271974	1.2599	11.713	0.6664	0.0000		SV	
7	4.623	154068	0.7137	7.810	0.4444	0.0000		VV	
8	5.023	154230	0.7145	4.580	0.2606	0.0000		VV	
9	5.897	29822	0.1382	1.652	0.0940	0.0000		VZ	
10	7.860	150572	0.6975	5.210	0.2964	0.0000		PP	
11	9.210	123419	0.5717	3.914	0.2227	0.0000		PP	
12	11.930	130807	0.6060	3.563	0.2027	0.0000		PV	
13	12.740	37712	0.1747	1.232	0.0701	0.0000		VZ	
14	15.103	504463	2.3370	11.048	0.6286	0.0000		PP	
15	16.390	143477	0.6647	3.512	0.1998	0.0000		PP	
16	19.350	56860	0.2634	1.827	0.1039	0.0000		PP	
17	23.510	806878	3.7379	11.274	0.6415	0.0000		PP	
18	27.167	92042	0.4264	1.224	0.0696	0.0000		PP	
19	30.633	144235	0.6684	2.243	0.1276	0.0000		PP	
20	33.747	239568	1.3414	3.947	0.2245	0.0000		PP	
21	38.727	250488	1.1604	3.231	0.1839	0.0000		PP	
22	41.340	61644	0.2856	0.771	0.0438	0.0000		PP	
23	43.727	29928	0.1386	0.470	0.0267	0.0000		PP	
24	57.047	112074	0.5192	9.096	0.5175	0.0000		PV	
25	57.433	175711	0.8140	10.275	0.5846	0.0000		VV	
26	57.907	107598	0.4985	4.508	0.2565	0.0000		VV	
27	58.137	25810	0.1196	2.279	0.1296	0.0000		VZ	
28	61.380	23245	0.1077	2.024	0.1152	0.0000		PV	
29	62.743	196612	0.9108	21.767	1.2385	0.0000		VV	
30	64.223	30411	0.1409	2.094	0.1192	0.0000		PV	
31	64.717	441196	2.0439	41.620	2.3681	0.0000		VV	
32	64.997	48524	0.2248	4.870	0.2771	0.0000		VV	
33	65.420	40354	0.1869	3.529	0.2008	0.0000		VV	
34	65.833	67766	0.3139	3.823	0.2175	0.0000		VZ	
35	66.950	21298	0.0987	1.691	0.0962	0.0000		PP	
36	67.773	32193	0.1491	1.708	0.0972	0.0000		PP	
37	79.013	29414	0.1363	0.920	0.0523	0.0000		PP	
38	97.297	21884	0.1014	0.456	0.0260	0.0000		PP	
39	100.053	38474	0.1782	0.738	0.0420	0.0000		PP	
40	104.457	126686	0.5869	2.358	0.1342	0.0000		PP	
41	107.010	57252	0.2652	2.113	0.1202	0.0000		PP	
42	111.397	35854	0.1661	4.236	0.2410	0.0000		VV	
43	111.813	28337	0.1313	2.512	0.1429	0.0000		VZ	
44	113.790	25408	0.1177	1.810	0.1030	0.0000		VZ	
45	115.883	43858	0.2032	3.808	0.2167	0.0000		PV	
46	119.590	25451	0.1179	1.656	0.0942	0.0000		PP	
total		21586234		1757.553		0.0000			

Table 3. HPLC spectrum figure of wild Zang Zhi

Table 3

No.	retention time	area (uv*sec)	area%	relative height	height%	conc	conc unit	baseline	ingredient name
1	1.550	1332009	0.5787	162.045	1.1488	0.0000		PV	
2	1.913	12708000	5.5210	797.430	5.6535	0.0000		VV	
3	2.607	7990504	3.4715	374.149	2.6526	0.0000		VV	
4	2.997	3336181	1.4494	151.464	1.0738	0.0000		VV	
5	3.480	827969	0.3597	90.536	0.6419	0.0000		VV	
6	3.687	2100942	0.9127	89.231	0.6326	0.0000		VV	
7	4.110	1665378	0.7235	80.468	0.5705	0.0000		VV	
8	4.590	986857	0.4287	61.543	0.4363	0.0000		VV	
9	4.867	1300649	0.5651	59.445	0.4214	0.0000		VV	
10	5.377	620409	0.2695	37.086	0.2529	0.0000		VV	
11	5.623	712514	0.3095	37.666	0.2670	0.0000		VV	
12	6.020	617412	0.2682	32.787	0.2324	0.0000		VV	
13	6.347	380678	0.1554	24.939	0.1768	0.0000		VV	
14	6.660	622333	0.2704	24.053	0.1705	0.0000		VV	
15	7.060	557302	0.2421	18.364	0.1302	0.0000		VV	
16	7.710	585765	0.2545	27.106	0.1922	0.0000		VV	
17	8.353	276221	0.1200	10.957	0.0777	0.0000		VV	
18	8.867	379887	0.1650	19.904	0.1411	0.0000		VV	
19	9.327	316284	0.1374	21.157	0.1500	0.0000		VV	
20	9.623	72943	0.0316	4.914	0.0348	0.0000		VV	
21	10.593	120581	0.0524	6.584	0.0467	0.0000		PP	
22	11.260	57846	0.0251	2.283	0.0162	0.0000		PV	
23	11.747	32476	0.0141	2.199	0.0156	0.0000		VV	
24	12.263	209326	0.0909	11.437	0.0811	0.0000		VV	
25	12.837	131315	0.0570	6.603	0.0468	0.0000		VV	
26	13.733	270732	0.1176	7.438	0.0527	0.0000		VV	
27	15.300	156380	0.0679	6.542	0.0464	0.0000		PP	
28	17.513	346774	0.1507	10.369	0.0735	0.0000		PV	
29	18.270	1011777	0.4396	25.904	0.1837	0.0000		VV	
30	19.863	298337	0.1296	7.175	0.0509	0.0000		VV	
31	21.567	237547	0.1032	6.715	0.0476	0.0000		VV	
32	22.657	261555	0.1136	7.129	0.0505	0.0000		VV	
33	23.960	419821	0.1824	8.165	0.0579	0.0000		VV	
34	25.780	1014237	0.4406	20.361	0.1444	0.0000		PP	
35	29.320	4482109	1.9472	103.960	0.7370	0.0000		PP	
36	32.017	6999710	3.0410	156.204	1.1074	0.0000		PV	
37	33.343	399151	0.1734	8.733	0.0619	0.0000		VV	
38	35.053	97125	0.0422	2.428	0.0172	0.0000		PV	
39	37.007	3062612	1.3305	46.150	0.3272	0.0000		VV	
40	39.587	832484	0.3617	15.926	0.1129	0.0000		PV	
41	40.897	2411303	1.0476	30.354	0.2152	0.0000		VV	
42	43.603	94412	0.0410	2.102	0.0149	0.0000		PP	
43	46.093	950329	0.4129	12.948	0.0918	0.0000		PP	
44	47.820	29686	0.0129	0.697	0.0049	0.0000		PP	
45	51.273	22016	0.0096	0.505	0.0036	0.0000		PP	
46	53.253	391511	0.1701	6.542	0.0464	0.0000		PP	
47	55.177	418679	0.1819	6.082	0.0431	0.0000		PP	
48	57.243	3988680	1.7329	240.883	1.7078	0.0000		PV	
49	57.500	4155318	1.8053	345.611	2.4503	0.0000		VV	
50	57.787	3545656	1.5404	257.829	1.8279	0.0000		VV	
51	58.010	1206951	0.5244	175.620	1.2451	0.0000		VV	
52	58.257	2986313	1.2974	222.955	1.5807	0.0000		VV	
53	58.400	5093407	2.2128	431.601	3.0599	0.0000		VV	
54	58.673	2540255	1.1036	286.541	2.0315	0.0000		VV	
55	58.943	5661787	2.4597	471.025	3.3394	0.0000		VV	
56	59.233	2400643	1.0430	197.347	1.3991	0.0000		VV	
57	59.543	3531683	1.5343	200.445	1.4211	0.0000		VV	
58	59.850	2228945	0.9684	164.165	1.1639	0.0000		VV	
59	60.060	2828869	1.2290	335.781	2.3806	0.0000		VV	
60	60.410	17547630	7.6235	994.818	7.0529	0.0000		VV	

61	60.743	13833800	6.0101	994.628	7.0516	0.0000	VV
62	60.920	9930270	4.3142	941.167	6.6726	0.0000	VV
63	61.343	3601150	1.5645	265.761	1.8842	0.0000	VV
64	61.723	6048523	2.6278	525.238	3.7238	0.0000	VV
65	62.127	2249845	0.9774	160.527	1.1381	0.0000	VV
66	62.357	2949680	1.2380	152.131	1.0786	0.0000	VV
67	63.207	11335680	4.9248	986.220	6.2830	0.0000	VV
68	63.460	3758168	1.6327	214.761	1.5226	0.0000	VV
69	64.087	17403430	7.5609	992.676	7.0377	0.0000	VV
70	64.807	6379895	2.7717	332.965	2.3606	0.0000	VV
71	65.487	1105522	0.4803	48.756	0.3457	0.0000	VV
72	66.060	1061718	0.4613	41.562	0.2947	0.0000	VV
73	66.490	1903467	0.8270	65.469	0.4642	0.0000	VV
74	67.563	1016818	0.4418	30.915	0.2192	0.0000	VV
75	68.387	1511594	0.6567	37.104	0.2631	0.0000	VV
76	69.390	10743430	4.6675	526.207	3.7306	0.0000	VV
77	70.583	358833	0.1559	13.087	0.0928	0.0000	VV
78	71.257	2732845	1.1873	127.212	0.9019	0.0000	VV
79	72.317	237862	0.1033	7.948	0.0563	0.0000	VV
80	72.730	245290	0.1066	6.784	0.0481	0.0000	VV
81	73.783	87523	0.0380	3.200	0.0227	0.0000	VV
82	74.250	127009	0.0552	4.136	0.0293	0.0000	VV
83	74.890	86115	0.0374	2.980	0.0211	0.0000	VP
84	75.973	28738	0.0125	1.165	0.0083	0.0000	PP
85	77.503	92275	0.0401	1.898	0.0135	0.0000	PP
86	80.060	508744	0.2210	11.408	0.0809	0.0000	PP
87	82.200	28158	0.0122	0.871	0.0062	0.0000	PP
88	84.293	79909	0.0347	1.534	0.0109	0.0000	PP
89	87.500	1092447	0.4746	24.011	0.1702	0.0000	VP
90	87.630	300046	0.1304	24.553	0.1741	0.0000	VV
91	87.893	382295	0.1661	25.603	0.1815	0.0000	VV
92	88.107	224523	0.0975	21.631	0.1534	0.0000	VV
93	88.397	717983	0.3119	37.094	0.2630	0.0000	VV
94	89.010	518852	0.2254	22.242	0.1577	0.0000	VV
95	89.237	307090	0.1334	21.551	0.1528	0.0000	VV
96	89.473	302635	0.1315	19.158	0.1358	0.0000	VV
97	90.007	4050733	1.7598	326.533	2.3150	0.0000	VV
98	91.020	376589	0.1636	16.743	0.1187	0.0000	VV
99	91.427	152969	0.0665	11.423	0.0810	0.0000	VV
100	91.643	205346	0.0892	11.033	0.0782	0.0000	VV
101	92.013	142333	0.0618	11.613	0.0823	0.0000	VV
102	92.157	268901	0.1168	11.479	0.0814	0.0000	VV
103	92.633	124915	0.0543	8.073	0.0572	0.0000	VV
104	93.083	908307	0.3946	88.376	0.6266	0.0000	VV
105	93.690	210904	0.0916	15.423	0.1093	0.0000	VV
106	94.063	323059	0.1404	22.173	0.1572	0.0000	VV
107	94.547	63428	0.0276	3.102	0.0220	0.0000	VV
108	95.123	29222	0.0127	2.059	0.0146	0.0000	VP
109	95.963	45135	0.0196	2.967	0.0210	0.0000	VP
110	98.950	58309	0.0253	3.644	0.0258	0.0000	VP
111	100.273	20525	0.0089	0.713	0.0051	0.0000	PP
112	105.563	38299	0.0166	1.804	0.0128	0.0000	PP
113	112.263	55710	0.0242	1.468	0.0104	0.0000	PP
114	126.417	44605	0.0194	0.861	0.0061	0.0000	PP
<hr/>							
total		230177556	14105.03	0.0000			

[0033] The following Analysis Examination and Pharmacology Examination demonstrate that according to the elements and functions of the concentrated product of the invention's cultured Zang Zhi fruiting body, its major elements include triterpenoid compounds, water-soluble polysaccharides and chitin.

Water Soluble Polysaccharides

[0034] Examination by the Food Analysis Center in Japan revealed that solid Zang Zhi fruiting body obtained by the invention's culture method contains a high volume of water soluble polysaccharides; each 100g fruiting body having 1.2 to 6.5g of water soluble polysaccharides.

Content of Heavy Metals

[0035] Atomic Spectrum Analysis made by the Microanalysis Laboratory of Atomic Science Department of National Ching Hwa University reveals the invention's solid-cultured Zang Zhi has a content of heavy metals as follows:

Na : 48.7ppm	Mn : 10.9ppm	Cd : ND
Mg : 767 ppm	Fe : 100 ppm	Cr : ND
Al : 41.6ppm	Zn : 16.4 ppm	
Ca : 605 ppm	As : ND	
K : 0.560%	Hg : ND	

Total Antioxidant Activity

[0036] A comparison among one gram of the concentrated powder of the invention's solid Zang Zhi substance, the powder of wild Zang Zhi, and the concentrated powder of *Ganderma* sp. reveals their equivalent of the number of micrograms of Vitamin C as follows:

<u>Source</u>	<u>Content of one gram powder /equivalent to the number of micrograms of Vitamin C</u>
Powder of wild Zang Zhi	633 ug ascorbic acid /g
Concentrated Powder of <i>Ganderma</i> sp.	854 ug ascorbic acid/g
Concentrated Powder of the invention's solid Zang Zhi substance	1180 ug ascorbic acid/ g

[0037] The result shows that the total antioxidant activity of the invention's solid Zang Zhi substance is far higher than that of the fruiting body of wild Zang Zhi and that of *Ganderma* sp.

Toxicity Test

[0038] According to the experiment of the acute oral toxicity test LD50, the tested mice were each fed with 2,000 mg/kg of the solid powder of the invention's Zang Zhi, while the other mice were fed with the specially distilled water as control. The results show that the tested mice neither act abnormally nor die. Hence, the volume of the acute oral toxicity test LD50 is higher than 2000 mg/kg.

Ames Test

[0039] Under the Ames test, the invention's Zang Zhi is examined by the plate mixed assay. Five tested bacterial isolates are *Salmonella typhimurium* TA97, TA98, TA100, TA102 and TA1535. Each isolate is under five concentrations analysis of 312.5, 625, 1250, 2500 and 5000 ug/plate, respectively. Coincidentally, a negative control group and a positive control group specific to bacterial isolate are included, and each group is triplicate. Test results show that negative control group falls in the acceptable range, and positive control group reflects an increase of mutant bacterial colonies. Whether or not the bacterial isolates are treated under big mouse liver enzyme metabolic system (S9), all tested concentrates do not show an obvious increase of colonies of Ames tested isolates, as shown in table 4.

Table 4 *Salmonella typhimurium* Ames Test

Test Target	Test examples	Feed dosage x Period	Test result
<i>Salmonella typhimurium</i>	Five mutant isolates required Histidine of TA97, TA98, TA100, TA102 and TA1535	tested concentration (ug/plate) 312.5, 625, 1250, 2500 and 5000 plate mixed assay for triplicate. The assay was under constant temperature $37\pm 1^{\circ}\text{C}$ for 48-72 hours, and the mutant colonies calculated.	Whether under big mouse liver enzyme metabolic system (S9) or not, all tested concentrations show no significant increase of Ames test mutant colonies. Test result is negative.

Anti-Lipid Peroxidation Ability Test

[0040] Anti-lipid peroxidation ability and anti free radical ability of the solid cultured Zang Zhi solid concentrate was tested by the lipid peroxidation in mouse brain

homogenization solution. Mouse brain homogenization solution exposed to an oxidative stress was stimulated by Fe^{2+} introduction and induced the kinetics of generation of lipid-derived free radical. Figure 2 shows that while increasing the concentration of triple and 8 fold the invention's Zang Zhi solid substance concentrate, it has a trend to inhibit lipid peroxidation and reduce the generation of free radical.

Protection of Liver Function Experiment

[0041] An experimental model using carbon tetrachloride to cause slow damage to the liver of a mouse to discover the effect of different concentrates of Zang Zhi on the slow damage to a mouse liver was established. Twenty-four mice (ICR strain) are divided into 4 groups as shown in Table 5.

Table 5. Zang Zhi Test Dosage and Animal Group Division

Group	Carbon Tetrachloride Dosage	Dosage	No. of Mice
A	0(control)	0(control)	6
B	40% CCl_4 /olive oil (0.3 mg/100g BW)	0(control)	6
C	As above	50 mg(triple)/Kg	6
D	As above	50 mg(8 fold)/Kg	6

[0043] The mice of Groups A and B to D were under a hypodermic injection of olive oil (0.1 ml/100g BW), and 40% of CCl_4 /Olive oil (0.3 ml/100g BW) twice a week. The mice of Groups A and D were fed with physiological saline through a stomach tube, while the mice of Groups C and D were fed with two different dosages of extract of the invention's cultured Zang Zhi for five weeks. Then, the eyepit blood of all mice was collected to examine the biochemical measurement related to liver function. After that, the tested mice were sacrificed and the largest liver leaf was taken out to proceed with the hitopathogenic observation, such as damage to the liver cells, change of lipid, necrosis, cellulose and degree of these changes, and so on. Biochemical tests include activities of GTP and GOT, which are conducted according to the standards of International Clinic Chemistry. The experimental results show that GTP and GOT in the blood of a mouse liver damaged by carbon tetrachloride obviously increased after five weeks. GTP of about 850 units is 20-22 fold above the normal, and GOT

of about 1700 units is 40- 42 fold above the normal (Fig 3-4). In addition, mice fed with triple and eight fold of concentrated extract of the invention's Zang Zhi have the capacity to inhibit the increase of GTP and GOT induced by CCl₄. Particularly, when the GTP and GOT approach the peak, the triple concentrated extract can inhibit the increase of GTP by 37% and the increase of GOT by 65%.

[0044] When comparing the liver tissue of a normal mouse with the liver tissue of the mouse fed on CCl₄ after six weeks, the liver tissue treated with CCl₄ conspicuously shows tumefacient and gray rough surface. However, the liver tissue of the mouse fed on different concentrated Zang Zhi extracts shows no tumefacient and gray rough surface. Besides, by staining, one can see the pile of lipid granules, the tumid liver cells around the middle vein, macrophage and other irritated white blood cells infiltrating the liver cell space, and partial necrosis cells showing nodular transformation. However, the liver tissue of the mouse fed on different concentrated Zang Zhi extracts has no pathogenic changes as described above.

[0045] Table 6 shows the semi-quantification analysis of pathogenic mouse liver tissue. N represents the normal group, D represents CCl₄ damaged group, X3 represents the group fed on triple concentrated extract, X8 represents the group fed on eight fold concentrated extract. It is proved that the concentrated Zang Zhi extract can significantly improve the pathogenic changes.

Table 6. Semi-Quantification Analysis of Mouse Pathogenic Liver Tissue

	Inflammation of liver	Fat degener- ation	Cell necrosis			Biliary Prolifer- -ation	Calcific -ation
			Local necrosis	Cosecutive necrosis	Tuberous transform- ation		
N	0	0	0-1	0	0	0	0
D	2-3	2-3	2	2	0-1	1	0-1
X3	1	1-2	0-1	1-2	0-1	0-1	0-1
X8	0-1	0-1	0-1	1	0	0-1	0-1

Cancer Cells Inhibition Experiment

[0046] The Food Industry Research and Development Institute is entrusted to conduct an experiment to find out how the Zang Zhi solid cultured concentrate of this invention can inhibit cell growth of cervix cancer (HeLa), stomach cancer (AGS), breast cancer (MCF- 7), liver cancer (HepG2), bowel cancer (COLO 320 HSR), etc. The results of cell growth inhibition are shown in Table 7. The Zang Zhi solid cultured concentrate of this invention can significantly inhibit the growth of tumor cells (up to 75% to 87%), especially the cells of breast cancer.

Table 7. Results of Cancer Cell Inhibition by Extract of Solid Cultured Zang Zhi

Cancer Cell Lines	Rate of Cell Growth Inhibition (%)		
	Average (%)	±	SD
Hela	83	±	3.1
AGS	84	±	2.7
HepG2	75	±	2.4
MCF-7	87	±	1.4

* The final concentration of the tested sample is 10% of the original concentration.

[0047] In addition, an experiment is conducted to find out the results of inhibiting bowel cancer growth by using 5 powder specimens (New1, New2, New3, New4 and New5), 11 liquid specimens (20L, New2, New3, 520, 521, 522, 523, 524, 525, 526 and 527) as well as their 1:10 specimen dilution by microculture tetrazolium assay. The results as shown in Figure 5 reveal that in the final concentration 1/10 of stock solution can at least inhibit 30% of cancer cell growth, and the specimen 20L can inhibit the growth of cancer cells up to 90%.

Analysis of Chromosome Mutation in Vitro

[0048] Further, an analysis of chromosome configuration mutation in vitro was conducted to treat Chinese hamster ovary cells with the invention's Zang Zhi. The cells at middle mitosis were analyzed to observe and measure the frequency of abnormal chromosomes. Analysis of the configuration mutation of chromosome can estimate the ability of the invention's Zang Zhi to damage the cellular chromosome. Three treatments are

given as follows: the first treatment is to add the invention's Zang Zhi to treat the cells for three hours, without adding the active mouse liver enzymes system (S9); the second one is to add S9 to treat the cells for 3 hours; and the third one is to treat the cells without adding S9 for 20 hours. All tested Zang Zhi concentrations under these three treatments cause no increase of the frequency of chromosomal configuration mutation. The experimental results are shown in the following Table 8.

Table 8. Analysis of Chromosomal Configuration Mutation

Experimental Target	Experimental Examples	Feed Period	Result
Chinese hamster ovary cells; ATCC, repository No. CCL-61 CHO-K1	Inoculate each 60 mm cell cultured plate according to the tested concentrations; each concentration has two plates; these samples are divided into three groups according to the different treatments.	<p>The tested concentrations of the first and second groups are 0, 312.5 625, 1250, 2500 and 5000 ug/ml.</p> <p>The tested concentrations of the third groups are 0, 30, 100, 1000, and 3000 ug/ml.</p> <p>After culture under different conditions, cells with scattered uniformly 18-21 chromosomes are selected for observation.</p>	<p>Results of the third group under treatment reveal that treatment of Chinese hamster ovary cells with the invention's Zang Zhi, before or after metabolic activities, does not show significant increase of the frequency of chromosomal configuration mutation of Chinese hamster ovary cells.</p> <p>The experimental result is negative.</p>

Analysis of Micronuclei in Animal Body

[0049] The clastogenicity of the invention's Zang Zhi in the animal body was examined. Further, the risk estimation of human hereditary toxicity was made according to the formation of micronuclei in reticular mouse red blood cells. Twenty-five mice were divided into 5 groups in this experiment. Analysis of the micronuclei reveals that the reaction of invention's Zang Zhi to circumference blood micronuclei of mouse is negative.

Table 9. Analysis of Micronuclei in Animal Body

Experimental Target	Experimental Samples	Feed period	Experimental Results
ICR mouse	Samples are: 1. Solvent control group 2. High dosage control group 3. Medium dosage control group 4. Low dosage control group 5. Positive control group 6. Each group has five mice.	1 st - 4 th groups of mice respectively were fed on 0, 500, 1000, 2000 mg/Kg dosage; Mice of positive control group were fed on 1.0 mg/Kg MMC. The blood was collected and the reticular red blood cells were observed under fluorescent microscopy.	1. There is no difference between tested dosage group and solvent control group. 2. The rate of reticular red blood cell analysis proves that high dosage would not be toxic to mouse marrow. 3. There is no tendency of dosage reaction to micronuclei rate and the comparison of the control group shows no significant increase. Thus analysis of the reaction of micronuclei of mouse circumference blood to Zang Zhi shows negative reaction.

[0050] According to the pharmacological data described above, the invention's solid Zang Zhi substance not only possesses active constituents similar to wild Zang Zhi, for example, polysaccharide and tertripoid, but also possess the same function of anti-lipid peroxidation, anti-free radicals and anti-oxidation. Besides, the invention's solid Zang Zhi substance possesses the function to protect the liver and fight against cancer.

[0051] While this invention has been described in detail with particular reference to its preferred embodiments, the principles and modes of operation of the invention have also been described in this specification. The invention should not be construed as being limited to the particular forms disclosed, which are illustrative rather than restrictive. Modifications, variations, and changes may be made by those skilled in the art without departure from the spirit and scope of the invention as described by the following claims.